Biochimica et Biophysica Acta 1803 (2010) 443–451

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

The oncofetal H19 RNA connection: Hypoxia, p53 and cancer

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ARTICLE INFO

Article history:
Received 7 July 2009
Received in revised form 24 November 2009
Accepted 13 January 2010
Available online 1 February 2010

Keywords:
Hypoxia
p53 tumor suppressor
H19 non-coding RNA
Hypoxia-inducible factor alpha
Cell viability

ABSTRACT

Expression of the imprinted H19 gene is remarkably elevated in a large number of human cancers. Recently, we reported that H19 RNA is up-regulated in hypoxic stress and furthermore, it possesses oncogenic properties. However, the underlying mechanism(s) of these phenomena remain(s) unknown. Here we demonstrate a tight correlation between H19 RNA elevation by hypoxia and the status of the p53 tumor suppressor. Wild type p53 ($p53^{wt}$) prevents the induction of H19 upon hypoxia, and upon its reconstitution in $p53^{null}$ cells. The last case is accompanied by a decrease in cell viability. The p53 effect is nuclear and seems independent of its tetramerization. Furthermore, using knockdown and over-expression approaches we identified HIF1-α as a critical factor that is responsible for H19 induction upon hypoxia. Knocking down HIF1-α abolishes H19 RNA induction, while its over-expression significantly enhances the H19 elevation in $p53^{null}$ hypoxic cells. In $p53^{wt}$ hypoxic cells simultaneous suppression of p53 and over-expression of HIF1-α are needed to induce H19 significantly, while each treatment separately resulting in a mild induction, indicating that the molecular mechanism of p53 suppression effect on H19 may at least in part involve interfering with HIF1-α activity. *In vivo* a significant increase in H19 expression occurred in tumors derived from $p53^{null}$ cells but not in $p53^{wt}$ cells. Taken together, our results indicate that a functional link exists between p53, HIF1-α and H19 that determines H19 elevation in hypoxic cancer cells. We suggest that this linkage plays a role in tumor development.

1. Introduction

Hypoxic regions are common in solid tumors and have an impact on tumor progression and on the therapeutic response. Tumor tissue hypoxia enhances angiogenesis, tumor development, and metastasis, and in unison, could lead to a failure of response to current therapeutic modalities [1]. A complex cellular network of gene products participates in these tumorigenic processes, serving to maintain cellular homeostasis, and thus enabling an adaptive survival under hypoxic stress conditions. We argue that the H19 imprinted gene is part of this cellular response, since we have recently found that the level of its RNA is up-regulated upon hypoxic stress [2].

The H19 gene does not code for a protein and is paternally imprinted and located close to the telomeric region of chromosome 11p15.5, an area that is frequently involved in pediatric and adult tumors [3,4]. While our understanding of the expression and imprinting of the non-coding H19 gene has progressed in recent years, its function remains enigmatic. Perhaps most intriguing has been the controversy over the function of H19 gene. Initially, H19 RNA was proposed to possess a tumor suppressive property based in its ability to suppress tumorigenicity [5]. It was reported that H19 gene expression was up-regulated *in vitro* in differentiating cells indicating a potential role of H19 RNA in differentiation [6]. In addition, loss of imprinting of the IGF2 gene and inactivation of the H19 gene have been implicated in the pathogenesis of embryonal tumors and Beckwith–Wiedmann syndrome [7,8]. Recently using multiple murine models of tumorigenesis it was shown that H19 acts as a tumor suppressor [9].

In contrast, a wealth of reports tightly link H19 expression to carcinogenesis [10–14]. Elevated H19 RNA was reported in both primary and metastatic tumors, in epithelial/mesenchymal transition (EMT), in migration and angiogenesis, in inflammatory diseases, and in wound healing (for a review see [15]). In addition, reports by others and our own have provided evidence that the H19 message possesses oncogenic properties [2,16–18]. Recently, we have shown that the H19 gene product is essential for human tumor development, and that this oncogenic activity of the H19 RNA is possibly enhanced upon hypoxic stress. Indeed, under hypoxic a condition, through as yet an unknown mechanism, the H19 RNA is significantly elevated [2]. In this study we delineate a tight correlation and functional link between H19 induction upon hypoxia and the presence of a functional p53 tumor suppressor gene product. Carcinoma cells respond to hypoxia

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doi:10.1016/j.bbamcr.2010.01.010

by up-regulating H19 RNA only in p53 mutated cells, and most severely in p53 null cells. The inhibitory effect of p53 on H19 elevation upon hypoxia is nuclear and seems independent of p53 transcriptional activity. Furthermore, we identify HIF1-α as a critical factor that is responsible for H19 elevation upon hypoxia and provided evidence that the p53 suppression effect on H19 may at least in part involve interfering with HIF1-α activity.

2. Materials and methods

2.1. Cell culture conditions and hypoxic treatment

All the cell lines used in this study and illustrated in Table 1 are grown essentially as described in [2], unless otherwise indicated. For hypoxic treatment different carcinoma cell lines were seeded in duplicates in 6-well plates in equal numbers. Twenty-four hours later cells were either placed into an aneoropack rectangular jar and supplemented with an anaeropack sachet (Mitsubishi Chemical Company, Japan) to create hypoxic conditions within an hour, or left under normal oxygen concentration. The hypoxic system creates a hypoxic environment of 0.1% O_2, 20% CO_2 within an hour. The progression of the hypoxic environment was monitored by a hypoxic indicator (Becton Dickson, Cock-eysville, MD, and U.S.A). Incubation lasted for an additional 24 h before RNA extraction.

2.2. RNA isolation, semi-quantitative and quantitative PCR (QPCR)

These procedures are performed essentially as described in [2,16]. PCR primer sequences, conditions and materials were described in [2,16]. For each specific case the numbers of PCR cycles are described in the legends of each figure. For HIF1-α semi-quantitative RT-PCR analysis, 1 μg total RNA was reverse transcribed as described in [16]. We used the following primer pair (5′-TCA CCA CAG GAC AGT ACA GGA TGC-3′ and 5′-CCA GCA AAG TTA AAG CAT CAG GTT CC-3′). The PCR reaction consists of 28 cycles carried out in the following manner: 94 °C for 1 min, 53 °C for 30 s, 72 °C for 40 s, with a final extension for 5 min at 72 °C and initial denaturation for 5 min at 94 °C.

Table 1
H19 induction triggered by hypoxic stress correlates with p53 tumor suppressor status. All the carcinoma cell lines are handled as described in Section 2. Shown is an overall summary of the results of semi-quantitative and quantitative RT-PCR reactions of H19 expression in hypoxic versus normoxic conditions, and its association with p53 status as deduced from the IARC TP53 mutation database and the p53 web sites. In the right column, the location of p53 mutations is depicted. The hypoxia system generates 0.1% O_2, 20% CO_2 within an hour.

Cell type[a]	Lineage source of carcinoma	P53 status	H19-modulation fold induction	P53 aa change
HepG2	Hepatocellular	Wild type	0.98	–
HepG2-2215	Hepatocellular	Wild type	1.05	–
FLC4	Hepatocellular	Unknown	16.2	UN
FLC4-A410	Hepatocellular	Unknown	26.3	UN
SNU-387	Hepatocellular	Mutated	3.6	164
SNU-475	Hepatocellular	Mutated	2.7	262
PLC/PRF/5	Hepatocellular	Mutated	3.8	249
Huh7	Hepatocellular	Mutated	2.8	220
Hep3B	Hepatocellular	Null	32	No expression
T24P	Bladder	Mutated	3.1	126
Umuc3	Bladder	Mutated	2.5	113
RT-112	Bladder	Mutated	Induced (ND)	248,183
JAR	Chorio	Wild type	1.09	–
JEG	Chorio	Wild type	1.04	–
OVCAR	Ovarian	Mutated	5.3	248
OV-90	Ovarian	Mutated	3.1	215
ES2	Ovarian	Mutated	8.2	UN
SKOV3	Ovarian	Mutated	3.8	89, 179
TOV112D	Ovarian	Mutated	6.5	175
Hela	Cervical	Wild type	1.1	–
MCF7	Breast	Wild type	1.5	–
4T1 (mouse)	Mammary	Null	13.3	No expression
A549	Lung	Wild type	1.03	–
H358	Lung	Null	15	No expression
H460	Lung	Wild type	No effect (ND)	–
H69AR	Lung	Mutated	Induced (ND)	171
HBE	Lung	Wild type	No effect (ND)	–
CC531 (rat)	Colon	Mutated	Induced (ND)	UN
HT29	Colon	Mutated	Not expressed	273

UN: Unknown. ND (not determined quantitatively).
[a] All cells are human except if specifically indicated.

2.3. Plasmid transfection conditions

The human full length $p53^{wt}$ and the mutant $p53^{NES-}$ ($p53^{L348A/L350A}$) expression vectors were obtained from Addgene. Mutant $p53^{NES-}$ plasmid was well characterized elsewhere [29,30].

Transfection of the p53 expression vectors in $p53^{null}$ (Hep3B) and (H358) cell lines was conducted with lipofectamine 2000 (Invitrogen, U.S.A.) in 12 well plates. The day prior to transfection, the cells were trypsinized, counted, and seeded at 90,000 cells/well containing 1 ml DMEM medium without antibiotics so that they were nearly 90% confluence on the day of transfection. Lipofectamine 2000 (3 μl) was incubated for 5 min with 100 μl serum-free OPTI-MEM medium (Invitrogen, U.S.A.) and supplemented with 1.6 μg of one of these plasmids ($p53^{wt}$, $p53^{NES-}$ ($p53^{L348A/L350A}$) an empty control plasmid) diluted in 100 μl serum-free OPTI-MEM media; the formulation lasted 20 min. 195 μl of the mixture was applied to the cells and incubated for another 24 h without replacement of the medium. After which cells were either placed in hypoxic conditions as described above or left under normal oxygen concentration for an additional 4 h before RNA extraction. Transfection of HIF1-α expression vector in $p53^{null}$ (H358) and $p53^{wt}$ (A549) lung carcinoma cell lines was performed as described above.

The level of activity of the co-transfected Luc 4 plasmid was used to normalize the differences in transfection efficiencies of the two p53 plasmids. For this purpose cells were co-transfected with the luciferase reporter (Luc4) and $p53^{wt}$ or $p53^{L348A/L350A}$ in a ratio of 1:5. Twenty-four hours post-transfection, whole-cell extracts from the transfectants were examined for luciferase activities following the protocol provided by the supplier (Promega). Differences in the luciferase activities when present were used to normalize the suppression effect of both p53 expression vectors on H19 RNA levels. A similar approach is used to normalize differences in transfection efficiencies of (H358) and (A549) cell lines with HIF1-α expression vector. Differences of the luciferase activities were used to normalize the induction effect of HIF1-α expression vector on H19 RNA levels.

2.3.1. In vivo model of H19 expression induced by different cell lines having different p53 status

2×10^6 carcinoma cells of the following lines: the human A549, H358, T24P, UMUC3, Hep3B and the murine 4T1 with different p53 status (Table 1) were suspended in 100 μl PBS and injected subcutaneously in the dorsa of 4 athymic mice, for each group. When the tumors reach about 8–12 mm in diameter, mice were sacrificed and RNA were extracted. For hypoxic indication, histological samples are taken from available tumors and fixed in formalin, processed, and embedded in paraffin. We stained 5-μm sections with hematoxylin–eosin and examined them by light microscopy for necrotic areas.

2.3.2. Cell viability assay

To test the effect of $p53^{wt}$ reconstitution on $p53^{null}$ cell viability reverse transfection was performed in 96 well plates with $p53^{wt}$ expression vector and an empty vector as a control using lipofectamine 2000 according to the protocol provided by the supplier. For each well, 0.25 μl of lipofectamine and 200 ng of plasmid were used. 1.5×10^4 (H358) cells were then seeded for 20 h after which cells were placed in hypoxic conditions as described for an

additional 4 h. MTT assay was performed according to the protocol provided by the supplier (Promega). The absorbance at 490 nm was measured using an ELISA plate reader.

To test the effect of H19 knockdown on cells viability, (A549) lung carcinoma and (Hep3B) hepatocellular carcinoma cells were exposed to hypoxia preceded by transfection with H19 siRNAs as described below. After 24 h in hypoxic condition cells were then washed twice with PBS, trypsinized and counted. 5×103 cells were seeded in quadruplicates in 96 wells and left to recover for 12, 24, 44 and 72 h at which time MTT assays were performed .The absorbance at 490 nm was measured using an ELISA plate reader.

2.3.3. Knockdown experiments

For H19 and HIF1-α mRNA knockdowns pre-designed stealth siRNAs together with a compatible negative control with nearly the same GC contents were obtained from Invitrogen, U.S.A. The knockdown experiments were essentially performed as described in [2]. For assessment of HIF1-α involvement in H19 induction upon hypoxia, $p53^{null}$ (Hep3B) cells were seeded in 6-well plates a day before transfection so that they were at 50% confluency. 100 pmol of three stealth siRNAs targeting HIF1-α and a negative control were formulated with 5 μl lipofectamine 2000 separately and transfected according to the protocol provided by the supplier. Four hours later cells were either exposed to hypoxic stress for an additional 20 h, or left under normal cell culture conditions before RNA extraction. The sequences of siRNAs targeting HIF1-α are as follows: HIF1-α stealth (1): CCA GCC GCU GGA GAC ACA AUC AUAU; HIF1-α stealth (2): AUA UGA UUG UGU CUC CAG CGG CUGG; HIF1-α stealth (3): GGG AUU AAC UCA GUU UGA ACU AACU; all written from 5′ to 3′ direction. For the H19 RNA knockdowns (A549) cells were transfected with H19 siRNA: CCA ACA UCA AAG ACA CCA U; and (Hep3B) cells were transfected with H19 stealth siRNA: CCU GAC UCA GGA AUC GGC UCU GGA A; written from 5′ to 3′ direction. For negative controls, (A549) cells were transfected with siRNA targeting luciferase [2] and Hep3B cells were transfected with negative control stealth siRNA having comparable GC contents with H19 stealth siRNA. Additional control are cells treated with lipofectamine 2000 alone (Mock). Twenty hours post-transfection, cells were placed in hypoxic conditions for 24 h. At this time point, the efficiencies of H19 knockdown was verified as described in [2] and MTT assay was performed as described above.

3. Results and discussion

3.1. Elevation of H19 RNA levels upon hypoxia is tightly linked to the status of the p53 tumor suppressor

Following our initial studies in which we showed an increase in H19 RNA level upon hypoxia in tumor cells [2], we questioned whether this phenomenon was a common feature of all tumor cells or whether this surge in H19 expression occurred only in distinctive types. Towards this aim, we initially focused our studies on a group of hepatocellular carcinoma (HCC) cell lines (nine such lines), and we observed very different patterns of response to hypoxia (Table 1). Some HCC lines showed no increase in H19 levels, some exhibited a

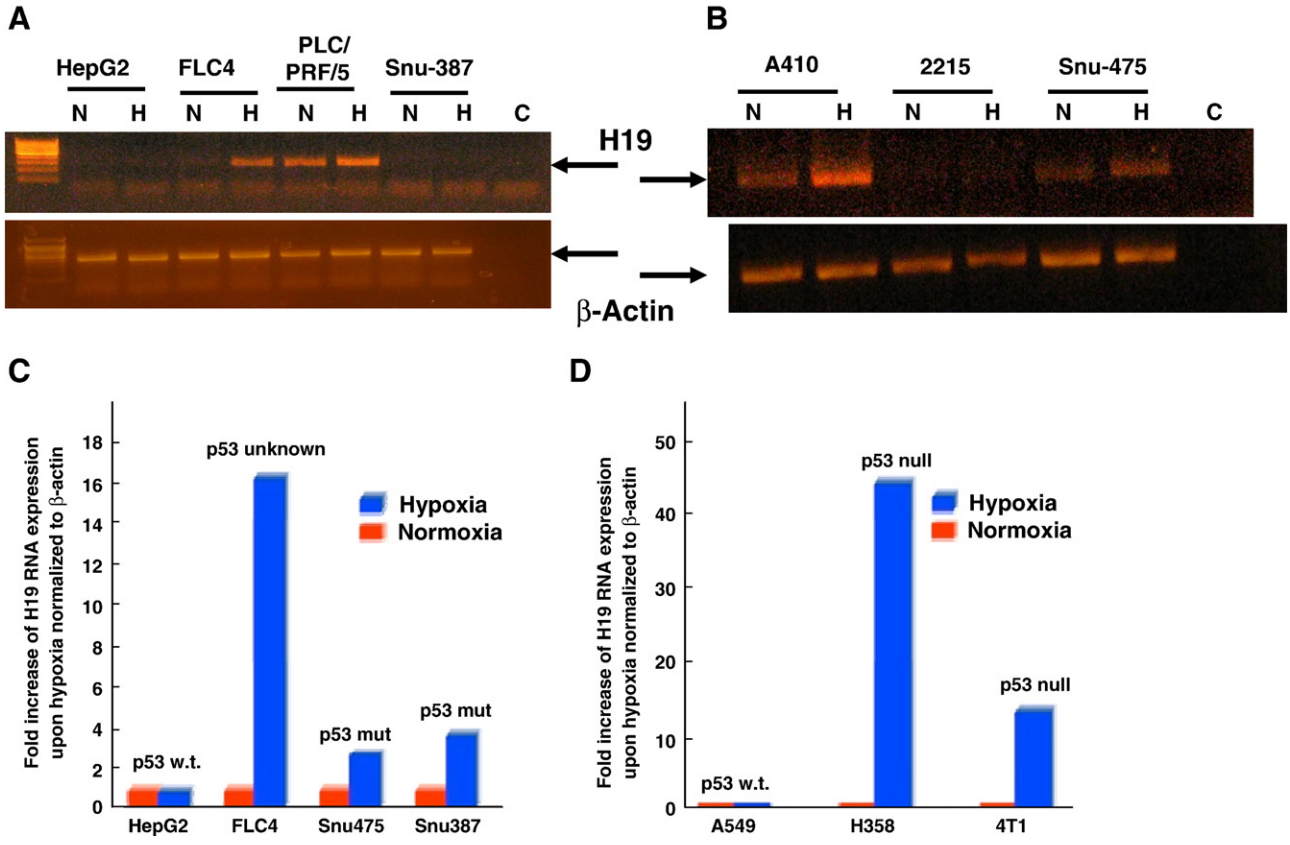

Fig. 1. H19 RNA level manifest a different induction pattern upon hypoxia in different carcinoma cell lines. Different carcinoma cell lines were cultured under normal conditions in duplicates for 24 h before hypoxic manipulation as described in the Section 2. (A, B): Semi-quantitative RT-PCR analyses of the H19 RNA (28 PCR cycles) cultured under normoxic condition (N), or hypoxic condition (H), for a panel of hepatocellular carcinoma cell lines. (A): HepG2; FLC4; PLC/PRF/5; and Snu-387; C is a PCR blank. (B): FLC4-A410, HepG2-2215, Snu-475. Shown under each figure are the semi-quantitative RT-PCR analyses of ß-actin mRNA to test for RT-PCR reaction integrity for the samples described above. Reversed transcription of total RNA was performed as described, except that 1 μg total RNA was used [16]. (C): For some of the samples described in parts A and B (HepG2, FLC4, Snu-387, Snu-475), QPCR analyses was performed to assess the levels of the H19 and ß-actin expression. (D): QPCR analyses of H19 and ß-actin were performed for human lung carcinoma (A549 and H358) and for the mouse mammary carcinoma (4T1) cell lines. For both C and D, the bars show relative modulations of the H19 RNA levels upon hypoxia relative to normoxia normalized to ß-actin values. These experiments were performed three times with similar results.

moderate increase, while others had a significant higher level of H19 RNA upon hypoxia. These results encouraged us to expand our investigation to include different lineage carcinoma cell lines ($n=28$), derived from lung, ovarian, bladder, chorio, cervical and breast, and to study H19 responsiveness to hypoxic stress. Similar to the different HCC cell lines, we observed a wide variety of responses towards hypoxia, ranging from no response, to a moderate response, or to a significant increase in H19 RNA level in the tumor cells (Table 1).

To gain insight into the possible mechanism associated with the different H19 gene response towards hypoxia, we searched for a common denominator among cell lines that do not elevate H19 RNA. The machinery controlling H19 imprinting and expression has become clearer in recent years [19,20]. Key cellular transcription factors were reported to control H19 transcription including c-myc, E2F1, Zac1, AFP regulator 1, FoxA , reviewed in [15,21] and the p53 tumor suppressor gene [22]. P53 was a leading candidate that we considered to be involved in H19 expression upon hypoxic stress. P53 lies at the protein complex hub of numerous signaling pathways that are induced in response to different forms of cellular stresses, including hypoxia [23]. In response to cellular stresses, p53 can either transactivate or transrepress its target genes and influence the cellular response to stress signals through transcription-dependent and independent pathways. Hypoxia is among the different non-genotoxic stresses that can activate the p53 tumor suppressor gene [24]. In particular, hypoxia induces nuclear p53 transrepression activity rather than transactivation [25].

We sought to investigate the association between the status of p53 in the different cell lines tested and H19 responsiveness to hypoxic stress, by exploring the vast data that is available on p53 sequence/phenotype in carcinoma cell lines, such as the IARC TP53 mutation database and the p53 web site {http://www-P53.iarc.fr/ and http://P53.free.fr/}. Our results revealed an interesting and strong correlation between the status of p53 gene and H19 RNA-induced expression in hypoxic stress. Out of the 28 cell lines studied, nine were $p53^{wt}$ and in all of those cells, H19 was not induced upon hypoxic stress, except for one cell line (MCF7) which we will elaborate upon below (Table 1 and Fig. 1). In contrast to $p53^{wt}$, $p53^{null}$ cells such as the human hepatocellular carcinoma (Hep3B), the human lung carcinoma (H358), and the mouse mammary carcinoma (4T1) cell lines, exhibited a high degree of H19 RNA levels induced by hypoxia (Fig. 1). The different $p53^{mt}$ cells on the other hand, showed a range of intermediate levels of H19 induction (Fig. 1). It is interesting to note that in the colon carcinoma (HT29) cell line, although possessing a mutated form of p53, it neither induces the H19 gene expression in response to hypoxic stress, nor expresses H19 under normal cell culture conditions (data not shown).

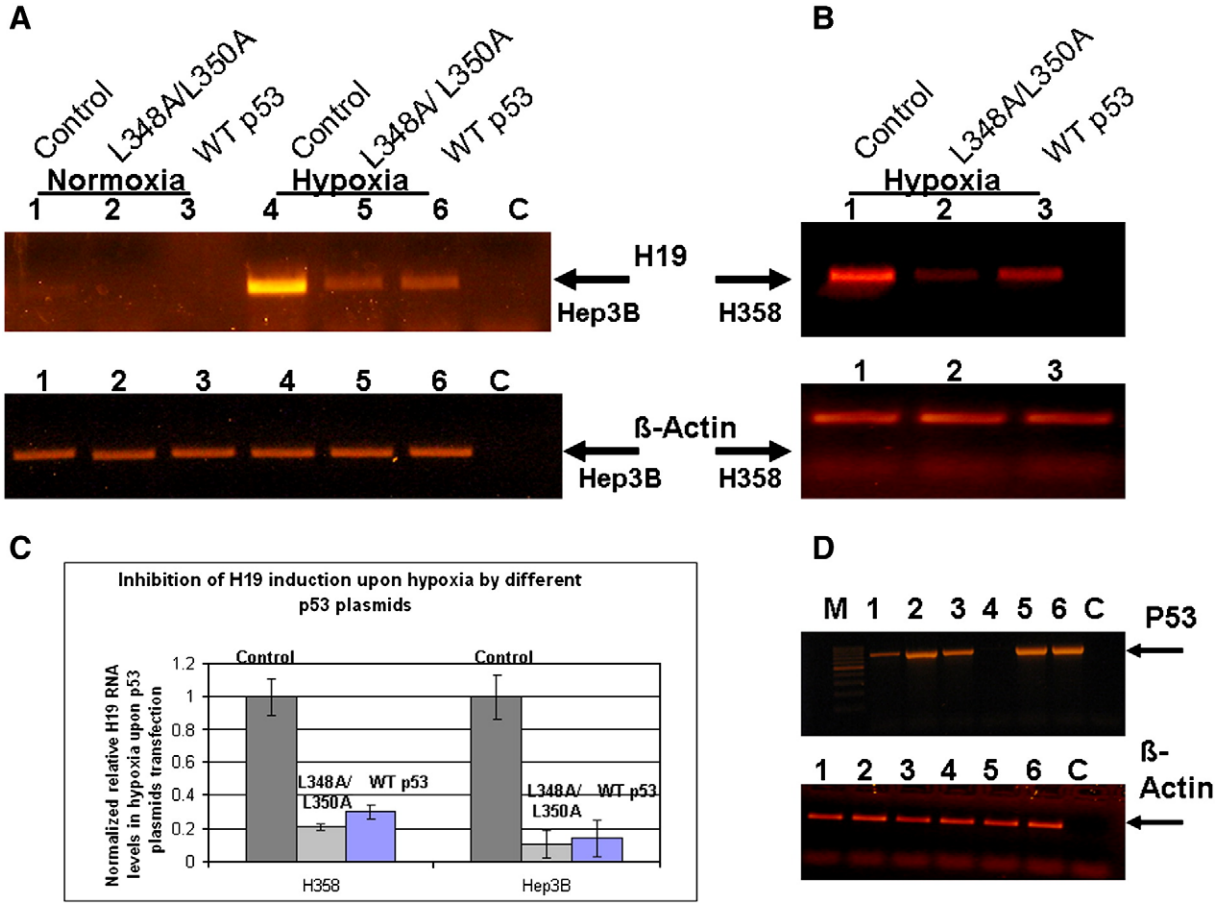

Fig. 2. P53 reconstitution through different expression vectors severely diminished H19 induction upon hypoxia and indicates nuclear effect. Hep3B and H358 cell lines both $p53^{null}$ were transfected with $p53^{wt}$ expression vector or ($p53^{L348A/L350A}$) expression vector harboring the L348A/L350A double mutation in which the nuclear exporting signal (NES) is abrogated. Shown (A): a semi-quantitative RT-PCR analyses of H19 RNA (29 PCR cycles) from (Hep3B) cells transfected with an empty vector as control, (lanes 1, 4), $p53^{L348A/L350A}$ expression vector (lanes 2, 5) and $p53^{wt}$ expression vector (lanes 3, 6). Shown are the expression levels of H19 in normoxia (lanes 1–3) and hypoxia (lanes 4–6). C is a PCR blank. (B): RT-PCR analyses of H19 expression upon hypoxia in (H358) cells transfected with an empty vector (lane1), $P53^{L348A/L350A}$ expression vector (lane 2) and $P53^{wt}$ expressing vector (lane 3). (C): QPCR analyses of H19 RNA levels upon hypoxia in the above samples. Shown are bars representing normalized values of H19 RNA levels (±standard deviations) in (H358) and (Hep3B) cell lines relative to control. The values are normalized to both β-actin expression levels and differences in transfection efficiencies of the plasmids. (D): Also shown are the expression levels of p53 mRNA in (Hep3B) cells (lane 1), and (H358) cells (lane 4) before transfection, and after being transiently transfected with $p53^{L348A/L350A}$ expression vector (lanes 2, 5), and $p53^{wt}$ expression vector (lanes 3, 6) respectively. M is a PCR ladder marker, and C is a PCR blank. The integrity of the RT-PCR reaction is verified by amplifying ß-actin gene product. These experiments were conducted three times at the least with similar results.

Altogether, these results may indicate that the presence of wild type p53 inhibits the elevation of H19 RNA under hypoxic stress.

3.2. $P53^{wt}$ reconstitution in $p53^{null}$ cells abolishes the induction of H19 RNA upon hypoxia

To further confirm that the elevation of H19 RNA in $p53^{mt}$ carcinoma cell lines triggered by hypoxic stress is directly related to p53 status, we transiently transfected two carcinoma cell lines harboring a $p53^{null}$ mutation (Hep3B and H358 cells) with a plasmid encoding the human $p53^{wt}$. Our results showed that upon transfection of $p53^{null}$ cells with $p53^{wt}$ expression vector, H19 elevation upon hypoxia was severely diminished (Fig. 2), revealing the existence of a tight link between the status of p53 and H19 responsiveness to hypoxia. This was accompanied by a decrease in cell viability as determined by MTT assay (Fig. 3A), in accordance with the known function of the p53 tumor suppressor.

3.3. The suppressive activity of p53 on H19 RNA elevation upon hypoxia is nuclear and seems to be independent on its transcriptional activity

P53 is known to function mainly as a transcription factor and therefore is dependent on its nuclear transport and retention [24,26]. The sub-cellular localization of p53 (nuclear versus cytoplasmic) is of great importance. Indeed, the activities of p53 in the cytoplasmic were shown to differ from its nuclear activities [27]. The H19 RNA is transported from the nucleus to the cytoplasm and associated with the ribosomes [28]. Determining the sub-cellular localization of p53 in hypoxia could shed light on the mechanism responsible for the p53 suppressive effect on H19 level.

P53 functions as a tetramer and it contains a highly conserved leucine-rich nuclear export sequence (NES) that is located within the tetramerization domain of the protein. Mutation of certain residues of this NES hampers both, tetramerization which is essential for its transcriptional activity and the export of p53 from the nucleus [29,30]. The p53 mutant $p53^{NES-}$ harbors the L348A and L350A double mutations in the NES region and is characterized by its inability to bind to HDM2 and by its exclusive nuclear localization, with a negligible cytoplasmic presence [29,30]. To assess the dependence of H19 hypoxic induction on the nuclear localization and the transcriptional activity of p53, we employed expression vectors carrying either the $p53^{wt}$ or the mutant $p53^{NES-}$ ($p53^{L348A/L350A}$) genes and transfected two p53-deficient cell lines, (Hep3B) and (H358) with these constructs. The expression of p53 from the plasmids was verified by RT-PCR analyses (Fig. 2D). Interestingly, compared to $p53^{wt}$, the mutant $p53^{NES}$ manifests comparable efficiency in suppressing the induction of H19 RNA upon hypoxic stress in both cell lines (Fig. 2A, B, C). The level of expression of the co-transfected Luc 4 plasmid was used to normalize the differences in transfection efficiencies of the two p53 plasmids (data not shown), and taken into account in calculating the quantitative PCR (QPCR) results (Fig. 2C). These results clearly indicate that p53 inhibits H19 RNA elevation in the nucleus. Moreover, this inhibitory effect seems to be independent of p53 tetramerization which is essential for its transcriptional activities. It is important to note that the only $p53^{wt}$ cell line that manifested a mild induction of H19 RNA upon hypoxia, was the

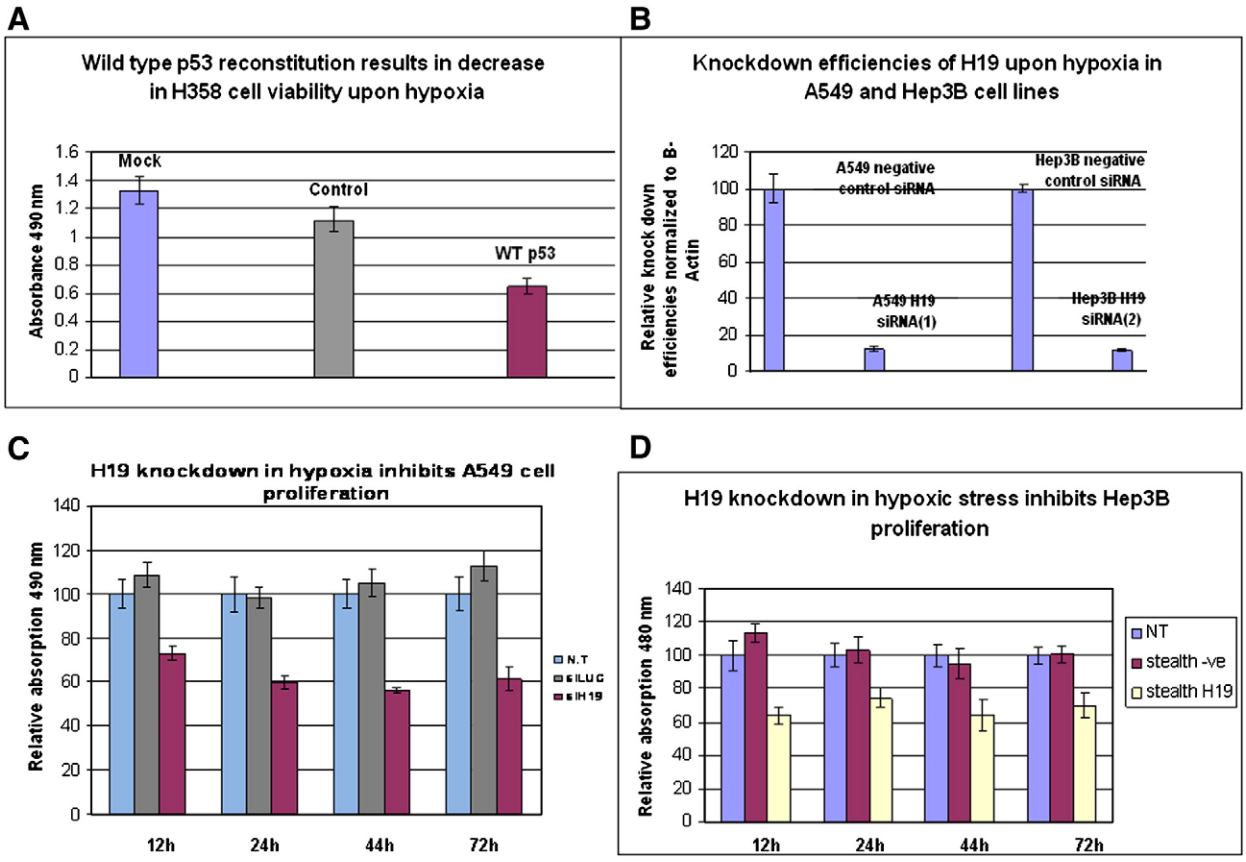

Fig. 3. Both p53 reconstitution and H19 RNA knockdown inhibit cells viability upon hypoxia recovery. The effect of the p53 reconstitution on cell viability was performed on (H358) cells by MTT assay as described in Section 2. Shown (A): the absorption values of the MTT solution from cells receiving lipofectamine 2000 without plasmid (Mock), empty vector as a control or $p53^{wt}$ expression vector. Every bar represents the mean ± standard deviation of four replicates. The effect of H19 knockdown on cell viabilities of (A549) and (Hep3B) after hypoxia recovery was performed as indicated in Section 2. Shown (B): knockdown efficiencies of H19 RNA determined by QPCR analysis, in hypoxic (A549) and hypoxic (Hep3B) cells. Two different H19 and negative control siRNAs (siLuc for A549 and stealth negative control for Hep3B) were used. (C and D): Relative absorption values of the MTT solution from non-transfected (N.T) cells, cells transfected with negative control siRNAs, and H19 siRNAs for (A549) are shown in (C) and for (Hep3B) are shown in (D). This was performed in different timescales following hypoxia. These experiments were repeated three times with similar results.

human breast carcinoma cell line (MCF7) (Table 1). Although these cells express $p53^{wt}$, the majority of the protein is rapidly excluded from the nucleus and accumulates in the cytoplasm [30]. This supports our observations that repression of H19 RNA elevation upon hypoxia necessitates the presence of a $p53^{wt}$ in the nucleus.

3.4. H19 knockdown upon hypoxia significantly decrease cell viability

Our previous observations indicate that neither H19 RNA knockdown nor its over-expression modulate the proliferative ability of the tumor cells under normal cell culture conditions [2,16]. However, over-expression of the H19 message confers a proliferative advantage to tumor cells grown in serum starved conditions [16]. This finding is in accordance with the observation that H19 RNA is needed for S-phase entry after serum starvation recovery, by E2F transcription factor binding to its promoter [18]. All these observations and our results in this report that show H19 RNA elevation upon hypoxia is p53 dependent suggest that H19 RNA is necessary for cell viability mainly under stress conditions, which are an integral part of tumor development. Accordingly we explored the effect of H19 RNA knockdown on the viabilities of hypoxic (A549) and (Hep3B) cells after being recovered for an extended timescale. The H19 knockdown experiments were performed using two siRNAs targeting different locations in H19 transcript, and were very efficient in knocking down H19 RNA in both hypoxic cells (Fig. 3B). Our results indicate that knockdown of H19 RNA upon hypoxia significantly decreases the viability of the (A549) cells (Fig. 3C). Similar results were also observed for (Hep3B) cells (Fig. 3D) using different H19 and negative control siRNAs ruling out the possibility that the observed phenotype is due to the off-target effects of the siRNAs used. These results are in accordance with our previous study that shows hypoxic cells that are devoid of H19 expression fail to form colonies in soft agar after being recovered as opposed to hypoxic cells that possess H19 RNA where both colony sizes and numbers are greatly reduced [2].

3.5. HIF1-α is critical for H19 induction upon hypoxia

We previously reported that H19 RNA enhances bladder carcinoma tumor growth and vascularization *in vivo* induced by $p53^{mt}$ cell line (T24P). Moreover we showed that $CoCl_2$ treatment, which is known for its ability to stabilize HIF1-α, up-regulates H19 RNA [2]. Given the crucial role of HIF-1α in hypoxic stress response, we next examined whether it is involved in the elevation of H19 RNA upon hypoxia.

We chose the hepatocellular carcinoma cell line (Hep3B) which manifests a great elevation of H19 RNA upon hypoxia (Table 1) and harbors $p53^{null}$ mutation as our model. Using knockdown approach three different stealth siRNAs targeting HIF1-α were transfected into the cells before hypoxic triggering. Semi-quantitative RT-PCR analysis verified siRNA mediated knockdown of HIF1-α message, of which two siRNAs were very efficient (Fig. 4B). Our results clearly indicate that efficient knockdown of HIF1-α severely diminished H19 RNA induction upon hypoxia as determined by QPCR analysis (Fig. 4A). The inefficient HIF1-α siRNA (Fig. 4B lane 4) shows minimal effect on H19 induction (Fig. 4A bar 4). The observation that the two siRNAs targeting different regions of the HIF1-α mRNA both diminished H19 RNA induction in hypoxic tumor cells demonstrate that this effect cannot be attributed to any of 'off-target' effects of the siRNAs used.

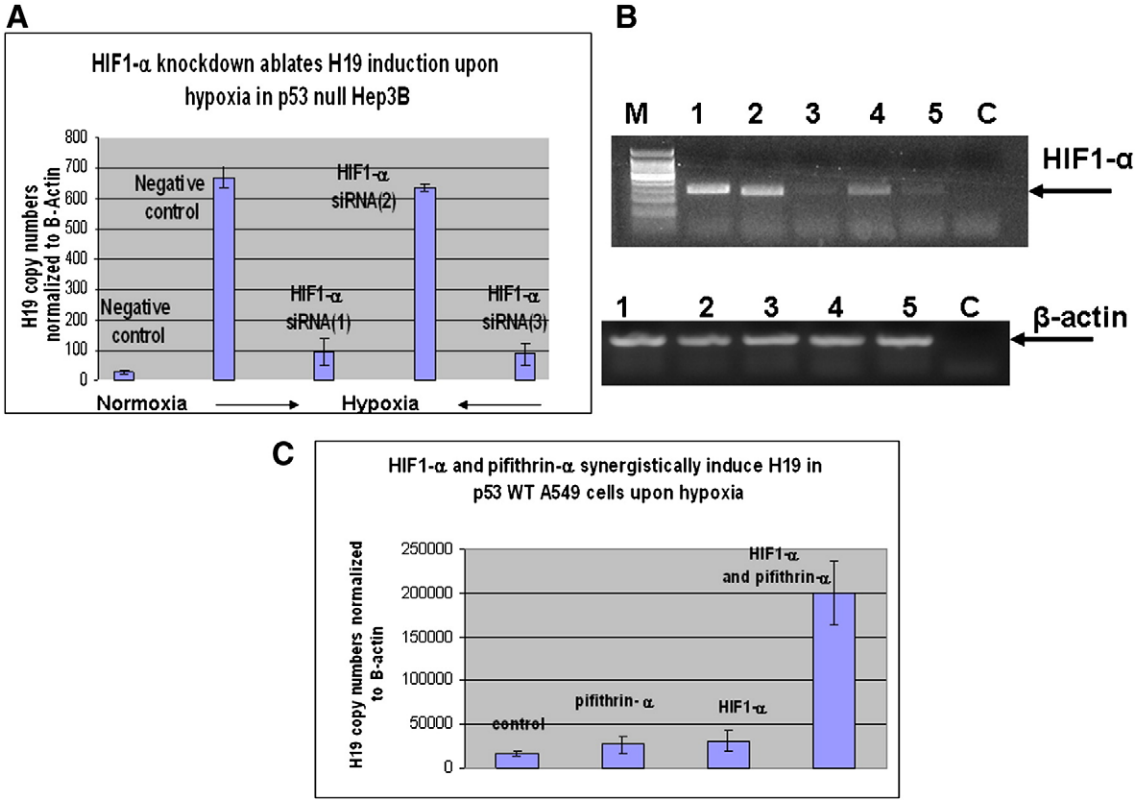

Fig. 4. HIF1-α is a critical factor responsible for the H19 RNA induction upon hypoxia. The $p53^{null}$ (Hep3B) cells were transfected with three different stealth HIF1-α siRNAs and a negative control siRNA as described in Section 2 before hypoxic treatment. A: QPCR analysis of H19 RNA levels normalized to β-actin in (Hep3B) cells in normoxic and hypoxic conditions previously transfected with a negative control stealth siRNA, and three different HIF1-α stealth siRNAs. Each manipulation is shown above the bars of the figure. B: The knockdown efficiencies of HIF1-α mRNA in the samples described above are verified by semi-quantitative RT-PCR analysis and show a very efficient knockdown of HIF1-α mRNA (lanes 3 and 5) treated with HIF1-α siRNAs 1 and 3, while HIF1-α siRNA2 (lane 4) is inefficient. This was compared to the levels of HIF1-α mRNA in normoxic (lane 1) and hypoxic (lane 2) cells transfected with negative control siRNA. (C): In $p53^{wt}$ (A549) cells, QPCR analyses of H19 RNA level normalized to β-actin show that simultaneous treatments of the cells with pifithrin α, and HIF1-α over-expression can synergistically induce H19 RNA upon hypoxia, while each treatment separately resulted in a mild induction. Each manipulation is shown above the bars of the figure.

To further confirm these results using over-expression approach, we transfect the human lung carcinoma (H358) cell line, harboring a $p53^{null}$ mutation, with HIF1-α expression plasmid under the transcriptional control of CMV promoter and explore its consequences on H19 RNA level in hypoxic cells. Again our results show that H19 RNA is induced in response to hypoxic stress as predicted, and that over-expression of HIF1-α significantly enhances this induction (Fig. 5).

Taken together, these results clearly demonstrate a critical role of HIF1-α on H19 RNA induction in hypoxic $p53^{null}$cells.

3.6. Simultaneous HIF1-α over-expression and p53 suppression synergistically induce H19 RNA level in hypoxic $p53^{wt}$ tumor cells

We thought to investigate if HIF1-α over-expression in $p53^{wt}$ hypoxic tumor cells, would relieve the inhibitory effect of p53 on H19. To this end we transfected (A549) lung carcinoma with HIF1-α expression plasmid as was performed above for (H358). Our results demonstrate that over-expression of HIF1-α could only mildly induce H19 RNA upon hypoxia on those cells (Figs. 4C and 5).

Several reports indicate that p53 inhibits hypoxia-inducible levels of HIF1-α by facilitating its ubiquitination and degradation [31,32]. This effect is a fundamental determinant of neoplastic growth and tumor progression by regulating angiogenesis [31]. Accordingly we explored if the mild effect of HIF1-α on H19 elevation described above would be enhanced by inhibiting p53 using pifithrin-α. Our results show that while neither HIF1-α over-expression nor pifithrin-α treatment could separately result in a profound induction of H19 RNA upon hypoxia, a simultaneous treatment significantly and synergistically induces the level of H19 RNA in those cells (Fig. 4C).

These results indicate that the inhibitory effect of p53 on H19 may at least in part be mediated through interference with HIF1-α activity under hypoxic stress. Indeed in all the $p53^{wt}$ cell lines tested in this study ($n=9$), $p53^{wt}$ prevents the increase of H19 RNA level upon hypoxia, but does not down-regulate its level below normoxic level (Table 1). This indicates that p53 inhibits the action of a transcriptional activator discovered in this study to be HIF1-α needed for H19 RNA induction upon hypoxia. Further investigations are needed to explore this point in more detail.

3.7. H19 RNA level is significantly elevated in tumors induced by $p53^{mt}$ cell lines in vivo

To explore the relevance of these *in vitro* data to the *in vivo* environment, we simulated hypoxic conditions by implanting a variety of carcinomas with different p53 status (Table 1) into the back of athymic mice and determined the levels of H19 RNA in the tumors that developed and compare it to its levels in those tumor cells *in vitro*. These are the $p53^{null}$ (H358) and $p53^{wt}$ (A549) human lung carcinoma cells, the $p53^{null}$ (Hep3B) human hepatocellular carcinoma cells, the $p53^{mt}$ (UMUC3 and T24P) human bladder carcinoma cells and the $p53^{null}$ (4T1) murine mammary carcinoma cells. As seen in (Fig. 6C, D), in a representative tumor generated from the $p53^{null}$ cells (H358), and (4T1) the level of H19 RNA was up-regulated, whereas in tumors formed from the $p53^{wt}$ cells (A549), H19 expression was significantly down-regulated. In both cases, regulation of H19 RNA level followed the same tendency as in the *in vitro* experiments but was more pronounced in the tumors (Fig. 6C, D). Up-regulation of H19 RNA *in vivo* was also seen from tumors induced from T24P, UMUC3 and Hep3B ([16], and data not shown). To assess the presence of hypoxic regions in the tumors *in vivo*, histological samples are taken and fixed in formalin, processed, and embedded in paraffin. We stained 5-μm sections with hematoxylin–eosin and examined them by light microscopy for necrotic areas. Representative samples taken from tumors induced by (UMUC3) and (T24P) reveal necrotic regions within the tumor (Fig. 6A, B) indicating that hypoxia is present.

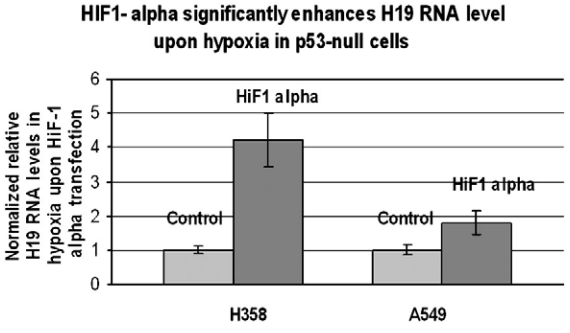

Fig. 5. HIF1-α significantly enhances the hypoxia responsiveness of H19 in p53-null cells. The human lung carcinoma (H358) and (A549) are $p53^{null}$ and $p53^{wt}$ cells respectively. Both cell lines are transfected with an empty vector as a control or a human HIF1-α expression vector as described in Section 2. The levels of H19 RNA were quantified using QPCR analyses. Shown are bars representing normalized values of H19 RNA levels (±standard deviations) in (H358) and (A549) cell lines transfected with HIF1-α expression vector relative to the control. The values are normalized both to β-actin gene product and the difference in transfection efficiencies of the two cell lines. These experiments were repeated four times with similar results.

It is generally accepted that hypoxia exerts physiological pressure that results in the expansion of tumor variants that have lost their apoptotic potential, and in particular variants that acquired p53 mutations [33,34]. A small number of transformed cells lacking $p53^{wt}$ could multiply and overtake cells expressing $p53^{wt}$ when exposed to hypoxia. $P53^{wt}$-expressing tumor cells are targeted for apoptosis during hypoxia [33]. Thus, p53 is a key regulator of cellular proliferation and survival associated with tumor progression during hypoxia [34].

Moreover, it was reported that HIF1-α is frequently over-expressed in many human cancers and there is a statistically significant correlation between the presence of mutant p53 and HIF1-α over-expression in biopsies of patients [35].

Here we show that tumor cells elevating the H19 RNA upon hypoxia also possess a mutation in the p53 gene. These same cells may also harbor enhanced HIF1-α activity resulting in a profound induction of H19 RNA upon hypoxia. Our earlier observation that H19 modulates genes whose expression is functionally involved in angiogenesis, survival and tumorigenesis in hypoxic stress indicates that H19 RNA could grant a selective advantage to tumor growth under stress conditions [2,16], which are important phenotypes resulting from p53 loss of function and HIF1-α increased transcriptional activity.

All the data presented in this study, and from our previous work [2,16], suggest a model predicting the role of p53 in H19 induction in hypoxic stress and tumorigenesis schematically presented in (Fig. 7). Under hypoxic conditions H19 RNA is elevated only in the presence of a mutated form of p53, and more severely in the complete absence of a functional p53 gene product. However, this scenario is blocked with the presence of wild type p53.

Taken into consideration that i) our finding that H19 RNA levels are elevated in hypoxic conditions in the presence of a mutated p53 gene, ii) the fact that many tumors carry mutations in p53, iii) H19 RNA is essential for human tumor growth [2] and iv) hypoxic conditions are common in tumor development, strongly suggest that carcinogenesis in p53 aberrant cells involves the expression of H19 RNA.

As hypoxia readily occurs in the majority of solid tumors driving critical steps in tumor development and metastasis and resistance to therapeutic modalities, it is of great interest to identify signaling pathways involved in this fatal outcome. In the light of our study, a molecular mechanism that integrates H19, p53 and HIF1-α to hypoxic stress response is uncovered. Placing the H19 gene product in this deadly circuit undoubtedly will have

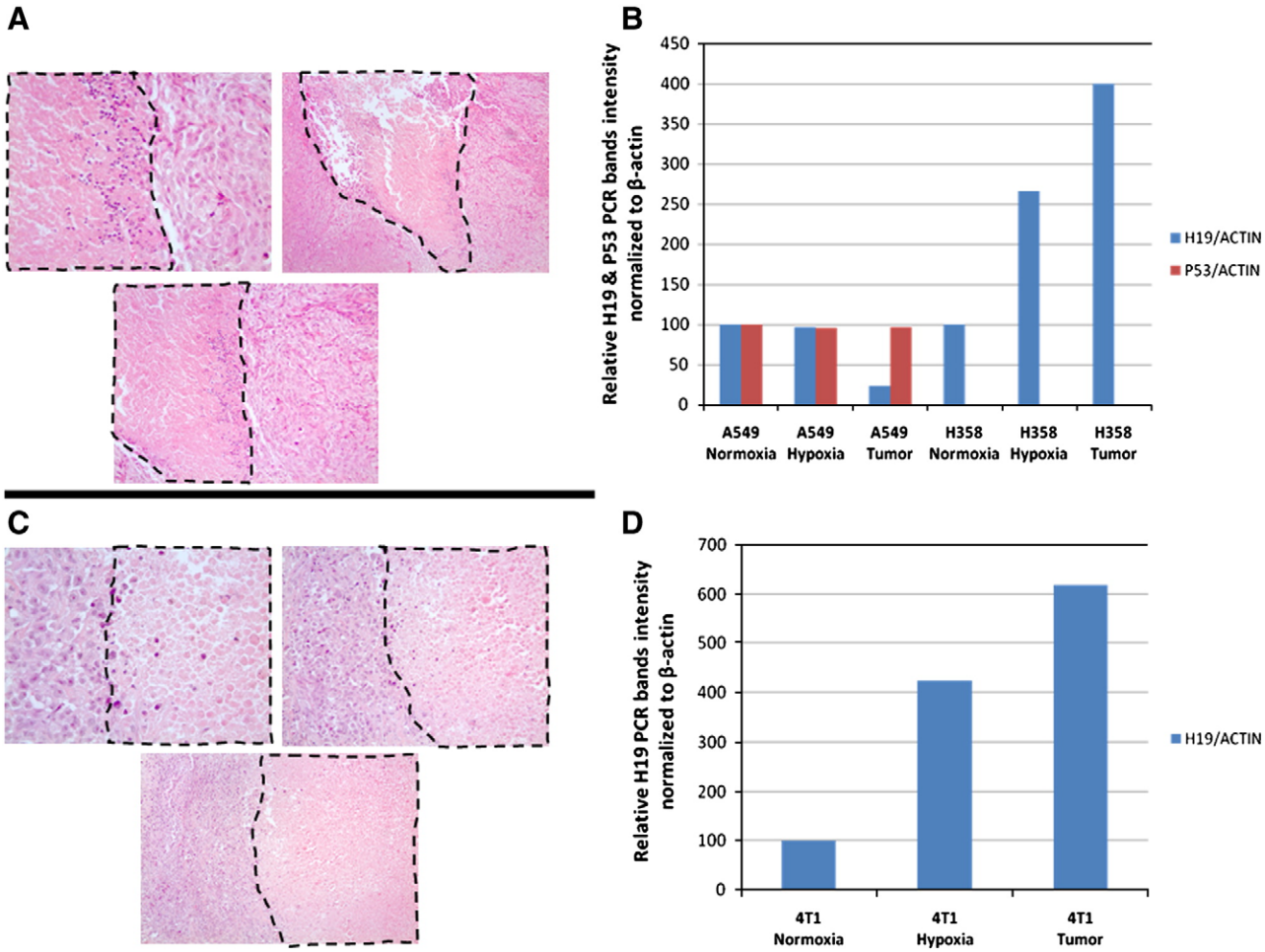

Fig. 6. P53 status correlates with the inducibility of H19 RNA *in vivo*. Cell lines with different p53 status were exposed to hypoxia or implanted subcutaneously into the back of athymic mice ($n = 4$ for each group). Twenty-four hours post hypoxia, or when tumors were formed, RNA was extracted for H19 QPCR analyses and histological samples for some of the samples were scored for necrosis. Shown are (A and B): necrotic areas indicative for hypoxia are highlighted by dotted lines in tumors that are induced from the bladder carcinomas (T24P) in A, and (UMUC3) in B. Shown is (C): a histogram presentation of semi-quantitative RT-PCR analyses of H19 RNA *in vitro*, in response to hypoxia, and *in vivo*, for (A549) and (H358) tumor cells respectively normalized to ß-actin (Blue bars). Results show an up-regulation of H19 RNA upon hypoxia and further up-regulation *in vivo* in H358 tumors. In A549, H19 RNA was not induced upon hypoxia as expected and with a significant down-regulation of its level *in vivo*. Also shown is a histogram presentation of semi-quantitative RT-PCR analyses of p53 mRNA (red bars) for A549 with different manipulations where p53 message showed no difference. (D): The h19 levels in murine (4T1) mammary carcinoma manipulated as described above also showed the same trend in up-regulating h19 RNA upon hypoxia and to a much stronger manner *in vivo*.

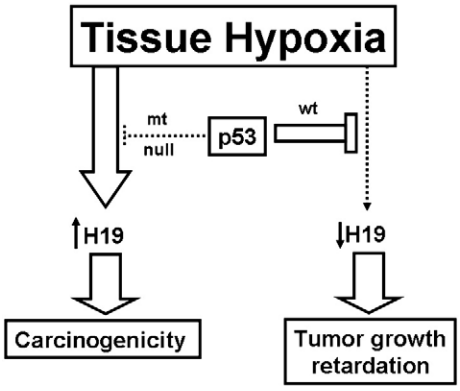

Fig. 7. Schematic representation predicting the role of p53 in H19 induction in hypoxic stress and tumorigenesis. Upon hypoxia, H19 transcription is highly induced, only in the presence of a non-functional p53 gene product. In the presence of functional p53, however, it blocks the induction of the H19 RNA through interfering with HIF1-α transcriptional activity. Since hypoxia is a major trigger for tumor progression, and H19 confers a selective advantage for tumor growth under stress conditions, and possesses oncogenic properties, these strongly suggest that tumor progression in p53 aberrant hypoxic cells involves the up-regulation of the H19 RNA.

major impacts in its utility as a target for cancer gene therapy. Indeed a DNA-based drug depending on H19 regulatory sequence and diphtheria toxin is now in clinical trial with promising preliminary results [15,21].

Further investigation is needed to decipher the mechanism(s) responsible for the increase in H19 RNA levels under hypoxic stress, and to explore the functional link between p53, HIF1-α and H19 gene products.

Competing interest statement
The authors declare that they have no competing financial interest.

Acknowledgements

This work is supported by the grants from the ISF, the FP6 program LSHB-CT-2004-512034 (MOLEDA), LSHB-CT-2005-018961 (INTHER) and the FP7 program LSHB-CT-2008-223317 (LIV-ES). Additional support was provided through grants from Barbara Fox Miller, Lille and Alfy Nathan, the Horowitz and the Wolfson Foundations.

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